ONLINE SUPPLEMENT

Unterweger et al: **Non-immunogenic dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs): A biocompatible, size-tunable contrast agent for magnetic resonance imaging.**

Materials and methods

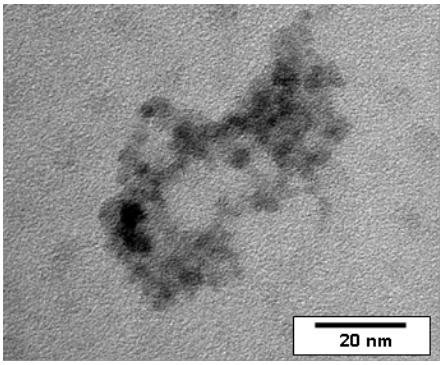
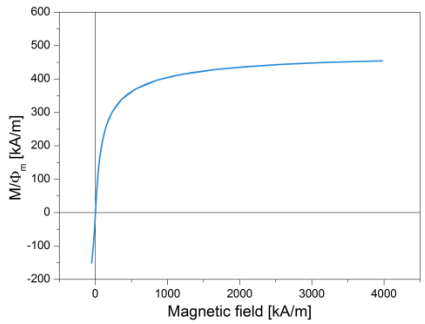
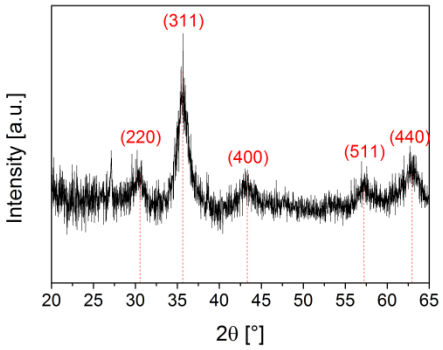
SPIONdex synthesis and characterization

The tested SPIONs (referred to as SPIONdex in the text) were synthesized according to a previously described method.[1] In short, $FeCl_2$ and $FeCl_3$ salts with a molar ratios of $Fe^{3+}/Fe^{2+} = 2$ were added to an aqueous solution of dextran T40. While stirring and keeping the solution under argon atmosphere, ice-cold 25% ammonia was added. Next, the particles were stirred for 40 minutes at 75°C, after which they were cooled to RT. The particles were then cleared from excess dextran and purified by dialysis and ultrafiltration. For the crosslinking (i.e. stabilization) of the dextran shell, epichlorohydrine was added, after increasing the pH with sodium hydroxide. The nanoparticles were then purified by dialysis against water and ultrafiltration. The final SPIONdex particles were then sterile filtered through 0.22 µm membrane.

For nanoparticle characterization (Table I), hydrodynamic particle size and ζ potential of SPIONs were measured with a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) as previously reported.[1] Transmission electron microscopy (TEM) pictures were taken with a FEI Tecnai 20 transmission electron microscope (Oregon, USA) with an acceleration voltage of 120 kV and a Gatan US 1000 CCD camera at 2 × 2 K resolution. For XRD measurements, a Bruker D8 Advance (Billerica, MA, USA) XRD was used. The magnetization of SPIONdex was measured using a SQUID-based susceptometer QD-MPMS-

XL-5. The magnetic field was increased from 0 to 4000 kA/m and subsequently decreased to -40 kA/m. SPIONdex sample shows superparamagnetic behavior (Table I).

Table I. SPIONdex characterization

TEM analysis	
SQUID analysis	
XRD analysis	

Particle sterility and endotoxin contamination assay

Quantitative determination of microbial contamination (quick agar plate test) - SPIONs were reconstituted in sterile water at concentration range 0.13-3.18 mg/mL. Sterile water was used as a negative control. For the positive control, bacterial cell culture (ATCC #25254) was used. Luria broth (LB) agar plates were streaked with samples and controls (50 µL) in duplicates and incubated for 72 hours at 37°C. To detect also low grade contaminations, particles were pre-incubated with cell culture medium without antibiotics for 10 days and sterility data were confirmed on Millipore yeast and mold sampler for 72 h (data not shown).

Kinetic turbidity Limulus Amebocyte Lysate (LAL) assay. to estimate levels of bacterial endotoxin - Assay range for kinetic turbidity LAL, which was used to determine the bacterial endotoxin concentration was from 0.001 to 1.0 EU/mL. For all steps, endotoxin-free reaction tubes and pipettes were used. LAL was reconstituted with Glucashield buffer (Associates of Cape Cod Inc., East Falmouth, USA), which prevents interference with beta-glucans by blocking the Factor G pathway of the endotoxin clotting cascade. In order to prepare the standard curves for calibration, specified amounts of USP-certified endotoxin standard (*E.coli* lipopolysaccharide (LPS)) were spiked into endotoxin-free (LAL-grade) water as previously described.[2] A set of quality controls was additionally included in every experimental test of each LAL assay. As a positive control, LAL-grade water spiked with 0.05 EU/mL endotoxin standard (i.e. standard curve midpoint concentration) was used. To prepare the inhibition/enhancement controls (IEC), 0.05 EU/mL endotoxin standard was spiked into the SPIONdex suspensions. As recommended by the FDA guidelines and USP standard for the LAL test, the validity of the results was accepted under the condition of precision and accuracy of the standard curve and quality control being within 25% and the spike recovery in IEC within 50-200%. At least three dilutions of the nanoparticles were tested whereby SPIONdex stock (3.18 mg Fe/mL) was diluted 1:5, 1:50 and 1:500. The diluted SPIONdex samples and their corresponding IEC were tested in duplicate. Endotoxin

analysis was confirmed using gel-clot Limulus Amebocyte Lysate assay whereby bacterial endotoxin reacts with an enzyme in the LAL leading to clotting of the lysate (Associates of Cape Cod, data not shown).

Blood stability test

SPIONdex particle suspension was mixed with freshly-drawn, rabbit whole blood (EDTA-stabilized), in order to reach a final iron concentration of 1 mg/mL. Water served as negative and lauric acid-coated SPIONs served as positive control. After 60 minutes incubation at room temperature, the vials were photographed. In parallel, 1 µL of each sample was smeared on a glass slide and photographed using a CCD camera connected to a light microscope (Zeiss Axio Observer Z1; Zeiss Optics, Jena, Germany). The results were confirmed with sheep whole blood (data not shown).

Hematocompatibility tests

Blood specimens from healthy humans were collected in EDTA tubes according to the Nanotechnology Characterization Laboratory (NCL, Frederick, MD, USA) protocol.

Complement activation – To assess the effects of SPIONdex on complement activation, the presence of complement split products was determined in plasma incubated with SPIONs. For this purpose, freshly drawn whole blood was centrifuged for 10 min at 2500xg to obtain platelet poor plasma (PPP). Plasma, pooled from three donors, was either used fresh or stored at -80°C until further use. For the experiment, pooled plasma was incubated with nanoparticles and veronal buffer (Boston BioProducts, Ashland MA, USA) for 30 min at 37°C (final nanoparticles concentrations 0-1000 µg/mL). As positive controls, cobra venom factor (Quidel Corp., Santa Clara, CA) and Cremophor-EL preparation (similar composition as in the clinical formulation of paclitaxel (Taxol), known to induce CARPA reaction) were used. PBS served as negative control. Plasma was diluted in complement specimen diluent

reagent (for iC3b: 1:1500 for positive control sample; 1:75 for negative control and other test samples; for C4d: 1:30 for all samples, and for Bb: 1:75 for all samples). To quantify the activation of complement in test plasma samples, the commercially available Quidel EIA kit was used to detect of the iC3b component of complement, as well as Bb (alternative activation pathway) and C4d (classical activation pathway), according to the manufacturer's instructions.

Plasma coagulation (prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT)) - To investigate whether SPIONdex influence plasma coagulation, platelet-poor human plasma from freshly-drawn whole blood anticoagulated with sodium citrate was obtained by centrifugation for 10 min at 2500 x g. Pooled plasma samples from 3 donors were treated with SPIONdex (0-200 µg/mL) for 30 minutes at 37°C. Subsequently, plasma was pipetted into pre-warmed cuvettes, a metal ball was added and coagulation was initiated by adding the respective coagulation activation reagent to each cuvette (Neoplastine for PT, $CaCl_2$ and PTT-A reagent for APTT, or thrombin for TT, all from Diagnostica Stago, Parsippany, US). Coagulation time was measured using a coagulometer (STArt4, Diagnostica Stago Gennevilliers, France). Standard plasma served as control for normal and abnormal coagulations.[3]

Platelet aggregation and ATP release - To investigate the effects of SPIONdex on platelet aggregation, whole blood from healthy volunteers was collected and anticoagulated with sodium citrate. Blood samples were centrifuged for 8 min at 200 x g in order to prepare platelet rich plasma (PRP) and for 10 minutes at 2500 x g to obtain platelet poor plasma (PPP) as a background control (blank). To ensure normal platelet function, all aggregation experiments were carried out within four hour period after blood collection. Each experiment was performed using plasma samples pooled from 3 different donors. PRP was incubated with SPIONdex, NaCl (negative control), or collagen (positive control) for 6 minutes at 37 °C.

Platelet aggregation (250×10^3 platelets/μL) was then measured in Model 700 aggregometer (Chrono-log Corporation, Havertown, PA, USA).[4]

Nanoparticle effects on normal human leukocytes

Isolation of PBMCs - Healthy volunteers' blood was collected according to the NCL protocol. Blood was drawn into tubes containing lithium-heparin. PBMCs were isolated using Ficoll-Paque® PLUS (GE Healthcare, WI, USA) from heparinized blood of three donors, according to the manufacturer's protocol.

Leukocyte procoagulant activity (PCA) – PCA, an *in vitro* indicator of disseminated intravascular coagulation was analyzed as described elsewhere.[5] Briefly, PBMCs (10.8×10^6 cells per sample in 3.6 mL RPMI medium with 10% FCS, 2 mM glutamine, 50 μM beta-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin) were incubated for 24 h with SPIONdex samples or water as negative control. As a positive control, a cocktail of LPS (10 μg/mL), PMA (phorbol myristate acetate, 50 ng/mL), and Ca-ionophore (10 μM) was used. After the incubation, cells were harvested, washed with PBS and reconstituted with pre-warmed (37°C) buffer A (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 0.15 M NaCl; 6.6 mM $CaCl_2$; pH 7.4). The equal volumes (0.1 mL each) of cell suspension and autologous plasma, prepared by centrifugation of freshly-drawn, sodium-citrate anticoagulated blood, were mixed. The coagulation time in quadruplicate samples was subsequently measured with a coagulometer (STArt4, Diagnostica Stago). Normal and abnormal coagulating plasma, as well as Neoplastin reagent served as controls.

Leukocyte proliferation assay - Isolated PBMCs from 3 different donors were adjusted to a density of 1 x 10^6 cell/ml in complete RPMI medium and incubated for 72 h with or without phytohemaglutinin (PHA-M, 10 μg/mL) with SPIONdex nanoparticles (0-500 μg Fe/mL). Subsequently, plates were centrifuged and nanoparticle-containing supernatant was removed. Afterwards, 150 μL fresh media were added to each well, together with MTT reagent (3-(4, 5-

dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), and incubated for 4 h, at 37°C. After the centrifugation, supernatant was aspirated and formazan crystals were solubilized using 200 µL DMSO and 25 µL glycine buffer per well. The absorption was analyzed spectrophotometrically at 570 nm.

Cell culture

Cell culture assays were performed according to the protocols established at the University Hospital Erlangen.

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords (kindly provided by the Dept. of Gynaecology, Prof. Beckmann, University Hospital Erlangen) using an established technique.[6] The use of human material was approved by the local ethics committee at the University Hospital Erlangen (review No.4449). Cells were cultured in Endothelial Cell Growth Medium (PromoCell, Heidelberg, Germany) with endothelial cell growth supplement containing 5% FCS, 4 µL/mL heparin, 10 ng/mL epidermal growth factor, 1 µg/mL hydrocortisone, 50 µg/mL gentamycin sulphate, and 50 ng/mL amphotericin B, at humidified 5% CO_2 atmosphere. In all experiments, HUVECs at passage 1-2 were used.

For THP-1 monocytic cell culture, RPMI 1640 medium was used. The standard medium was supplemented with glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal calf serum (FCS). Viability of cells was assessed using Trypan blue exclusion method and was greater than 98%. THP-1-derived macrophages were obtained by treating THP-1 cells with 20 ng/mL phorbol myristate acetate (PMA). The resulting THP-macrophages were harvested, washed and used for experiments 24 h later.

Human primary tubular epithelial cells (hTEC) were kindly provided by Prof. Goppelt-Struebe (Dept. of Nephrology, University Hospital Erlangen). These cells were isolated from renal cortical tissues, which were collected from healthy parts of kidneys of patients

undergoing tumor-nephrectomies, as described previously[7]. To facilitate cell attachment, hTEC were cultured in medium containing 2.5 % FCS for 24 h before the experiments. Afterwards, medium was replaced with epithelial cell selective DMEM/Ham's F12 medium (supplemented with 2 mM L-glutamine, penicillin/streptomycin, insulin-transferrin-selenium supplement, 10 ng/mL epidermal growth factor, 36 ng/mL hydrocortisone and 4 pg/mL triiodothyronine) without FCS.[7] To identify tubular cell populations enriched in proximal or distal tubular cells, the presence of N-cadherin or E-cadherin, respectively was detected by immunostaining. Cells were used at passage 2.

Monocytic cell recruitment under flow conditions

The y-shaped flow-through cell culture slides were obtained from Ibidi® (Munich, Germany). Numerical flow simulation[6] at a flow rate of 9.6 mL/min identified the region of laminar shear stress (10.2-10.8 dyne/cm^2) throughout the straight main channel, and the region of non-uniform shear stress at the outer walls of bifurcation (with shear stress ranging from ~6.3 dyne/cm^2 to ~0.5 dyne/cm^2).

HUVECs were seeded in the bifurcating slides at 7x10^5/mL and grown until confluence. The resulting cell monolayer inside the y-shaped slide channel was perfused with medium (with 0-400 µg/mL SPIONdex) for 18 h, at the physiologic arterial shear stress (10 dyne/cm^2, corresponding to flow rate of 9.6 mL/min), using a programmed peristaltic pump (Ismatec, Wertheim, Germany). After 18 h, HUVECs were stimulated with TNF-α for 2h, followed by perfusion with for 1 h with medium containing 7x10^5 cells per mL. After 1 h adhesion assay, non-adherent THP-1 monocytic cells were removed by washing with PBS. Adherent monocytic cells were fixed with 4 % paraformaldehyde, stained with hematoxylin and eosin (Dako, Hamburg, Germany), and counted in 8 image fields (0.89 mm^2) at x100 magnification in non-uniform shear stress area using Zeiss Axio Observer.Z1 microscope (Carl Zeiss AG, Germany) and ImageJ software.

Endothelial cell migration assay

Spontaneous migration of HUVEC was investigated using in a modified barrier assay. For this purpose, silicone cell culture inserts from Ibidi (Munich, Germany) were used as previously described.[8] HUVECs (at a concentration of $3x10^5$/mL) were seeded in 2 insert wells separated by a 500 µm barrier and allowed to adhere for 6 h. The cells were subsequently treated with 0-100 µg/mL SPIONdex particles overnight. On the following day, the inserts were removed and cells were washed, followed by the incubation with fresh medium containing appropriate amount of particles for 24 h. The Incucyte FLR live-cell microscopy system was used to monitor the gap size between the 2 monolayers at the insert removal point (0 h), and after 12 and 24 hours. Cell-free areas on recorded images at these time points were subsequently measured using ImageJ software. To determine whether SPIONdex affect cell migration, the increase in the area occupied by cells at 12 h and 24 h was calculated and compared with the cell-covered area at 0 h between particle-treated and untreated samples.[8]

Monocytic cell chemotaxis

The effect of SPIONdex (0-400 µg/mL) on the chemotactic behaviour of monocytic cell was investigated using a 96-well Chemo-Tx plate (NeuroProbe, USA), according to the manufacturer's instructions, whereby the microplate wells were filled with serum-free RPMI 1640 (30 µL/well). THP-1 monocytic cells were incubated with SPIONdex for 2h at 37°C, under constant stirring. As positive control, MCP-1 (50 ng/mL) was added to the specified wells. After placing the filter frame on the microplate, the filter top sites with 5 µm-pores were filled with 25 µL of nanoparticle-treated THP-1 cells at a concentration of $1x10^6$ cells/mL. The cells were allowed to migrate for 1 h at 37°C, followed by collecting the

migrated non-adherent cells from the lower wells, fixation and counting by flow cytometry. The samples were run in quadruplicate.

Analysis of complement activation-related pseudoallergy (CARPA)

The analysis of infusion reaction to SPIONdex was carried ou in a pig model, according to the protocols established at the Semmelweis University, as previously described by Szebeni et al. [9] As a study animal, male Yorkshire pigs weighing approximately 20-25 kg were used. Prior to the experiment, the animals were anesthetized with isoflurane, followed by intubation to maintain free airways. Oxygen saturation, $EtCO_2$ and the respiratory rate, as well as rectal body temperature were continuously monitored. Pulmonary arterial blood pressure (PAP) was measured in the pulmonary artery using a Swan-Ganz catheter (Arrow International, Inc., Athlone, Ireland) and the systemic arterial blood pressure (SAP) in the femoral artery. Saline (negative control), SPIONdex and zymosan (positive control) were administer by bolus injection (<10 s) through the left external jugular vein. Monitoring of hemodynamic changes (PAP, SAP and heart rate (HR) data) was started prior to the test material administration, continued for 10 m at average 20 s in every minute, and subsequently followed every 5 min until the end of the reaction to the test substances. ELISA kit for TXB2 (Cayman Chemicals, USA) was used to analyze the plasma concentrations of thromboxane B2, the stable metabolite of thromboxane A2, upon administration of test substances. The study protocol was approved by the local Animal Ethics Committee.

Histology

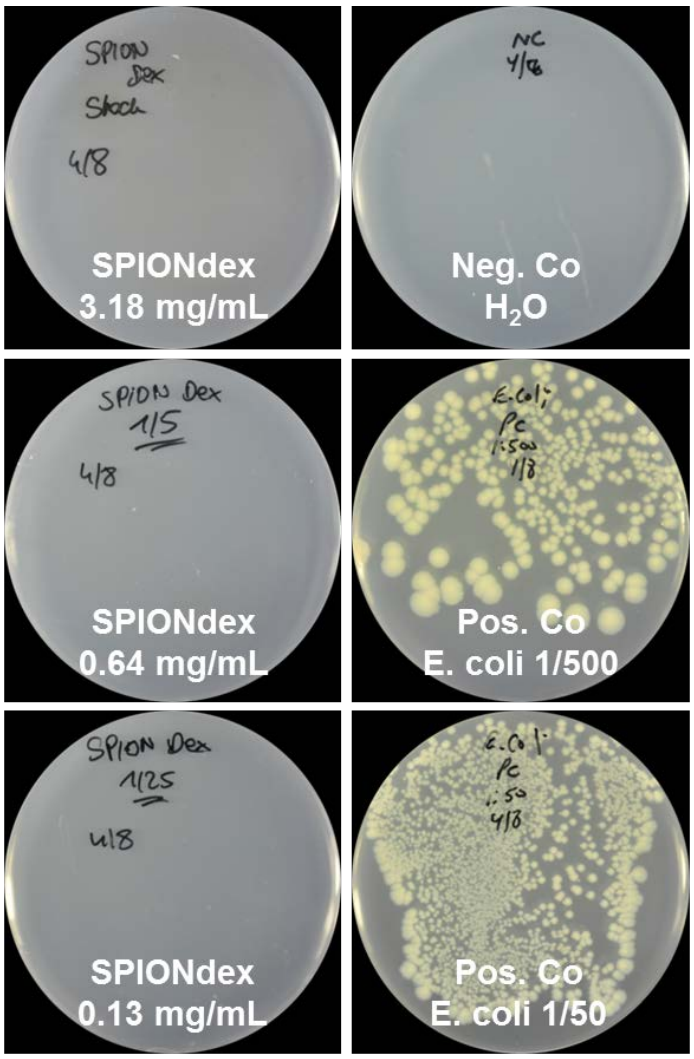
Liver tissue was stained with Prussian blue to assess the presence of SPIONdex. Briefly, the livers were fixed in 4% formaldehyde solution in PBS for 2 days. Afterwards, samples were dehydrated in an ascending isopropanol sequence and finally embedded in paraffin. Blocks were cut using the microtome and the resulting serial sections of 4-µm-thickness were

subsequently dewaxed in xylene, rehydrated in ethanol, and immersed in 1:1 solution of hydrochloric acid (2%) and potassium ferrocyanide (2%) for 30 min at RT. Cell nuclei were counterstained with liquid Fast-Red, followed by brief briefly with distilled water. Mowiol (Sigma-Aldrich) was used as a mounting medium. Images were taken using Axio Observer.Z1 microscope (Zeiss, Germany).

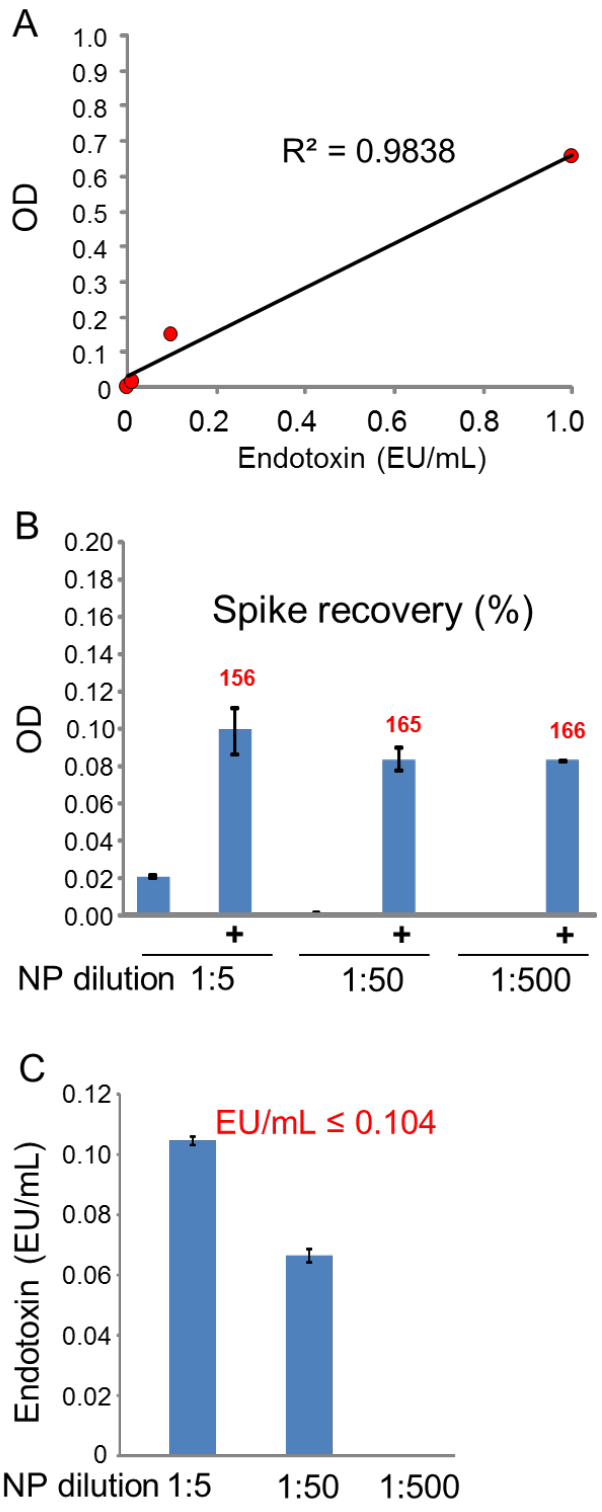
Supplementary Results and Figures

Sterility

To confirm the lack of microbial contamination, quick agar sterility tests (Suppl. Fig. 1), as well as the kinetic turbidity LAL assays for the presence of endotoxin, were performed. Bacterial endotoxin reacts with an enzyme in the LAL leading to turbidity. Higher endotoxin concentrations result in shorter onset times of turbidity, thus allowing for calculating the endotoxin level in the sample. As shown in the Suppl. Fig. 1 and 2, SPIONdex were free of bacterial contamination, with endotoxin levels below 0.105 EU/mL. These results were also confirmed by LAL gel-clot assay, whereby bacterial endotoxin reacts with an enzyme in the LAL leading to clotting of the lysate (not shown).



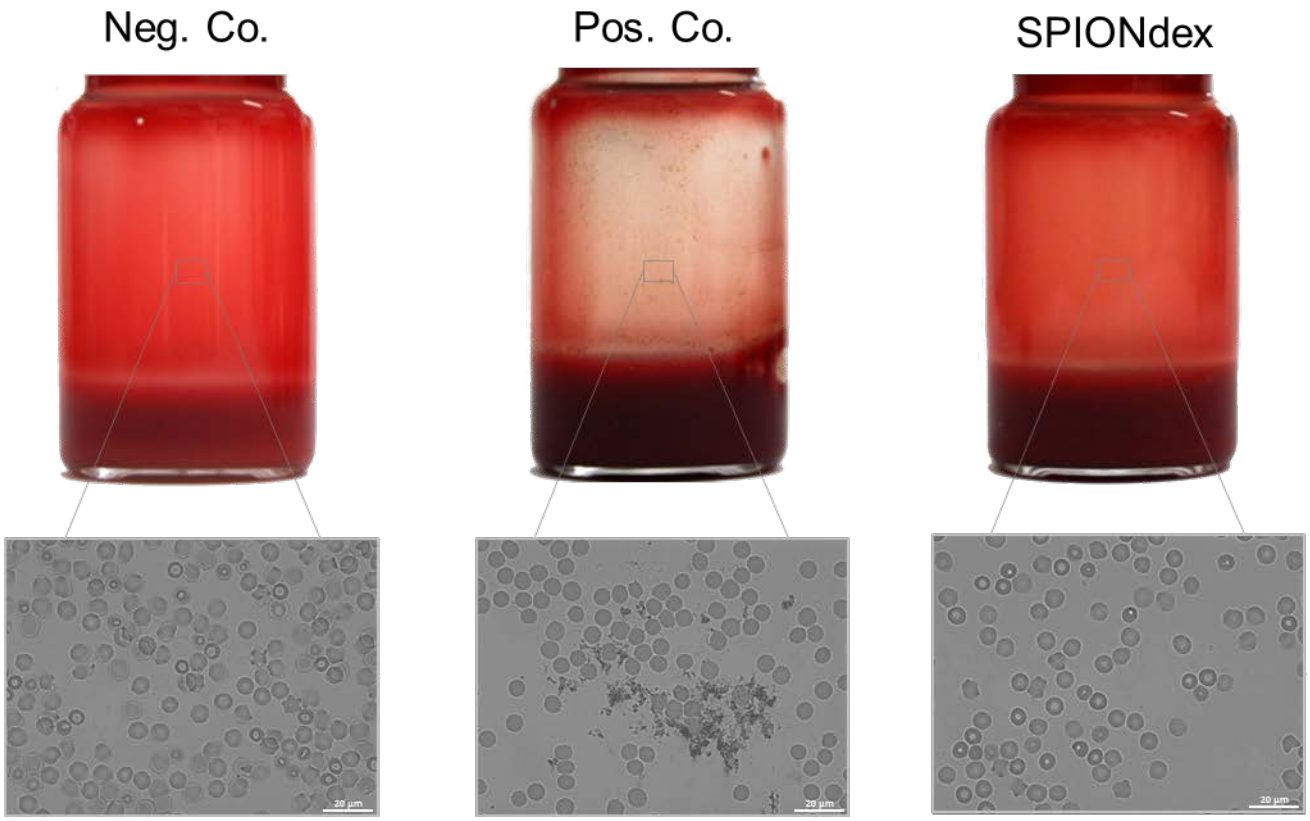
Suppl. Figure 1. Agar plate sterility test.
SPIONdex particles were reconstituted in sterile water at concentration range 0.13-3.18 mg/mL. For the positive control, bacterial cell culture was used. LB agar plates were streaked with samples and controls (50 µL) in duplicates and incubated for 72 hours at 37°C.



Suppl. Figure 2. Kinetic turbidity LAL assay for the presence of endotoxin.
(A) Endotoxin standard curve (1, 0.1, 0.01, 0.001 mg/mL);
(B) Spike recovery analysis in the SPIONdex-treated samples: **+**, samples spiked with 0.05 EU/mL endotoxin;
(C) Calculated endotoxin levels.

Blood stability

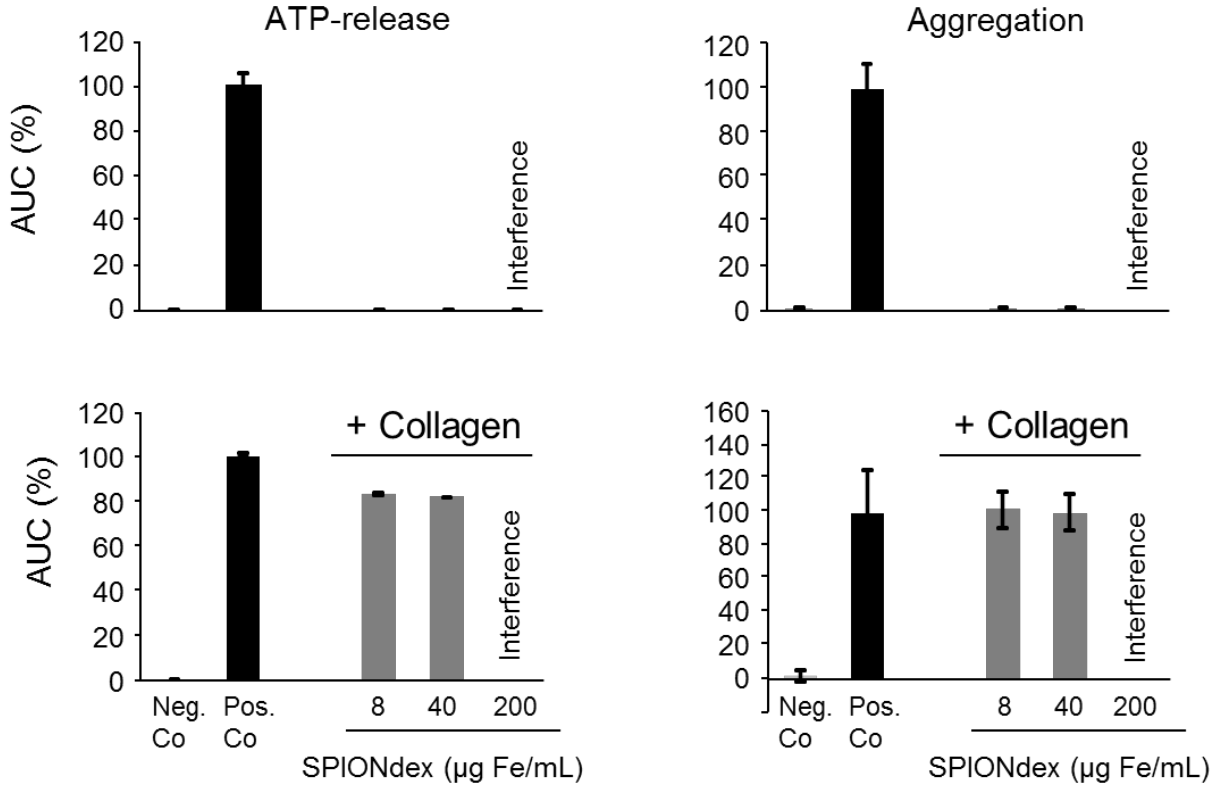
The blood stability of SPIONdex was evaluated macro- and microscopically using EDTA-anticoagulated rabbit whole blood (Suppl. Fig. 3). SPIONdex particles were stable and exhibited neither macro- nor microscopically detectable aggregates over the whole observation period (60 min). Since the detection limit of the used microscope is approx. 200 nm, it can be concluded that incubation with SPIONdex did not lead to formation of major aggregates above 200 nm. These results were confirmed with sheep whole blood (data not shown).



Suppl. Figure 3. Blood stability assay.
Investigation on the blood stability of SPIONdex in EDTA-anticoagulated rabbit whole blood. Blood sample was mixed with SPIONdex to achieve the iron concentration of 1 mg/mL. After 45 min incubation, images were recoded using bright light microscope. Pos. Co: lauric acid-coated SPIONs.

Platelet aggregation

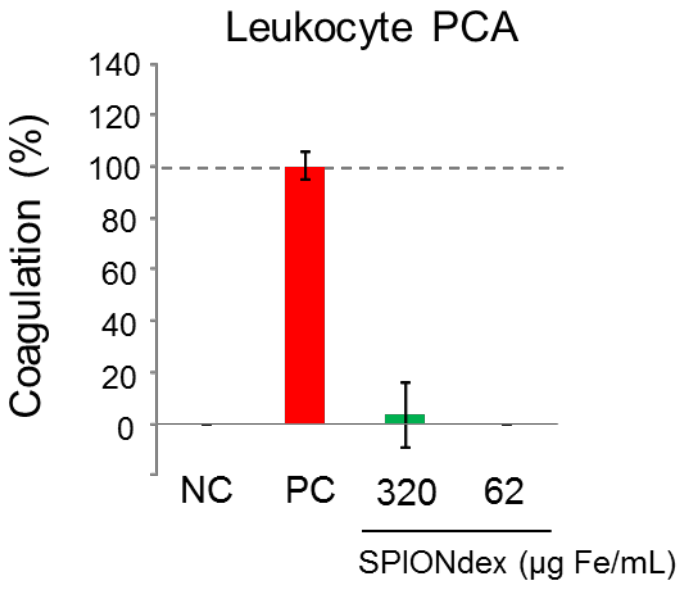
In unstimulated samples, the treatment with SPIONdex had no effect on platelet aggregation or ATP release (Suppl. Fig. 4, upper panel). However, at the highest nanoparticle concentration tested in this assay (200 µg/mL), an interference with the light-scattering-based measurement was observed resulting from the presence of SPIONs. SPIONdex did not affect the collagen-stimulated platelet aggregation and activity (Suppl. Fig. 4, lower panel).



Suppl. Figure 4. Effects of SPIONdex on platelet activation.
Platelet aggregation and ATP release in SPIONdex-treated samples. PBS served as negative and collagen as positive control. Means ± SD of replicate samples pooled from 3 donors are shown.

Leukocyte procoagulant activity (PCA)

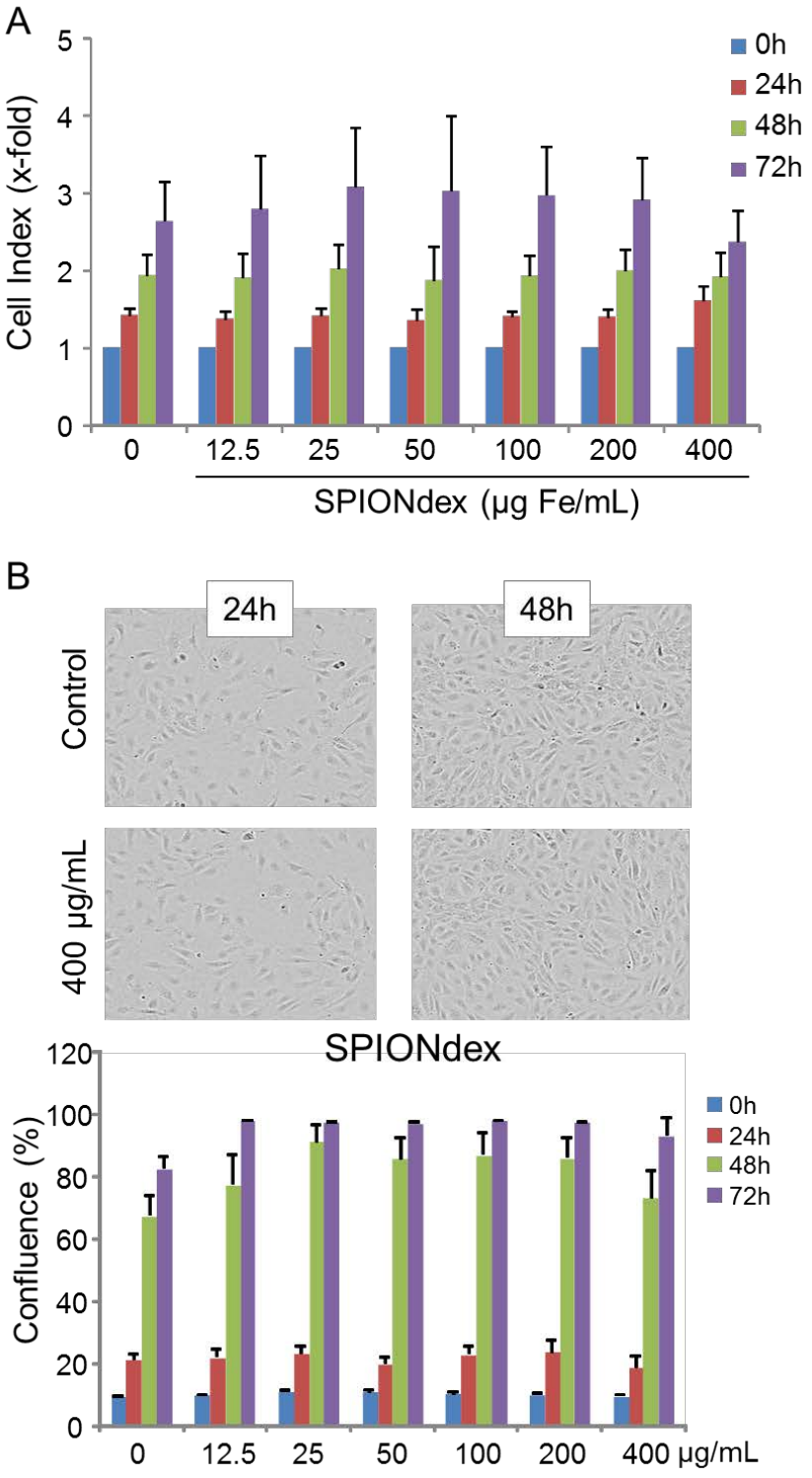
Intravenous injections of engineered nanoparticles in mice have been linked to fatal coagulopathy,[10,11] resulting from nanoparticle-induced presentation of phosphatidyl serine-tissue factor complex on the surface of leukocytes (PCA), which triggers activation of the extrinsic plasma coagulation cascade.[12-14] To exclude the induction of PCA by SEONdex, plasma coagulation time was measured in samples mixed with PBMCs pre-incubated with SPIONdex for 24 h. As shown in Suppl. Fig. 5, no effect of SPIONdex on PCA was detectable up even at the highest assayed concentration (320 µg/mL).



Suppl. Figure 5. Leukocyte Procoagulant Activity (PCA).
PBMCs were incubated for 24 h with test SPIONdex samples or water as negative control (NC). As a positive control (PC), a cocktail of LPS, PMA and Ca-ionophore was used. Following the incubation period, PBMCs were washed and mixed with autologous plasma. The measurement of coagulation time was done using a coagulometer.

Effects on vascular cell function

Endothelial cell viability in SPION-treated samples (0-400 µg/mL) was monitored for 72h using real-time cell analysis (i.e. impedance-based technique, xCELLigence, Roche) and live cell microscopy (IncuCyte FLR microscope, Essen Bioscience), as reported before.[15] Briefly, the treatment with SPIONdex for up to 72h had no influence on the viability or the morphology of HUVECs, even at the highest nanoparticle concentration (400 µg/mL), as compared with SPIONdex-untreated controls.[15] Similarly, in the flow-based assay using y-shaped flow-through slides, the endothelial monolayer exposed to the SPIONdex at 400 µg/mL for 18 h remained intact, without any alterations in cell morphology or confluence (Suppl. Fig. 6,[15]). Concerning cell motility, no differences in migration between control and SPIONdex-treated HUVECs were detected (data not shown).

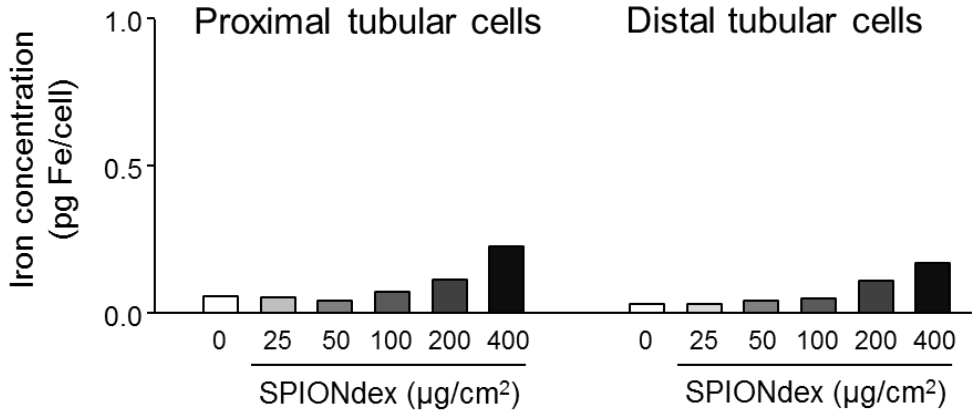


Suppl. Figure 6. SPIONdex effects on endothelial cell (EC) viability.

(A) Cell index measured by real time cell analysis. HUVECs were seeded at $1x10^3$ cells per well and grown for 24 h. Afterwards, medium containing SPIONdex was added to the wells. Cell index was recorded for 72 h. (B) Cell morphology evaluated using a live cell-imager (IncuCyte FLR microscope system. HUVECs were seeded in 96-well plates at $2x10^3$ cells/well and grown for 24 h. Afterwards, medium containing SPIONdex was added to the wells for 72h. The x10 objective magnification images were used to quantify the confluence levels using ImageJ.

Nanoparticle internalization by cells

To investigate whether SPIONdex biocompatibility/bio-inertness results from their low cellular uptake, we also used an *in vitro* model system based on human cells characterized by entirely different endocytotic capacities. This system comprises proximal tubular cells with high endocytotic potential, and distal tubular cells characterized by a very low internalization of larger molecules or particles.[16] Our studies in this model showed that although SPIONdex uptake was slightly increased in proximal compared to distal tubular cells, the overall uptake of these particles was very low, with cellular iron levels below 0.25 pg/cell in proximal and below 0.2 pg/cell in distal tubular cells after 24 h incubation with 400 µg/cm^2 (Suppl. Fig. 7).



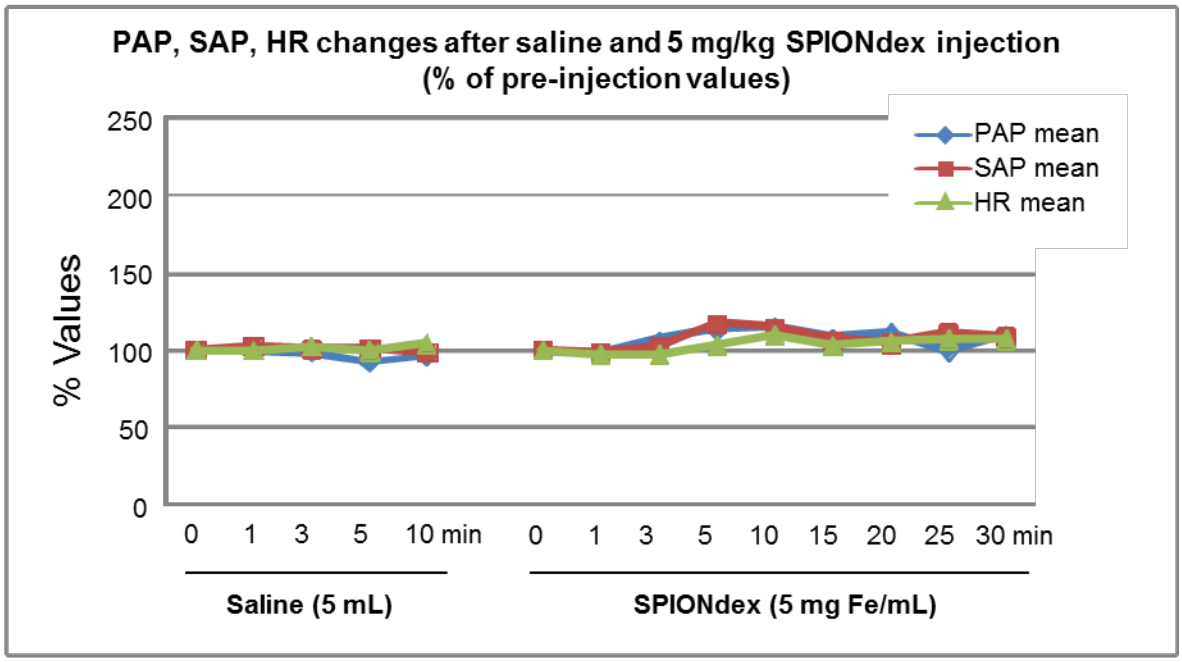
Suppl. Figure 7. Internalization of SPIONdex by tubular cells.
hTEC were grown to 90% confluence, followed by 24 h incubation with the indicated SPIONdex concentrations. Iron content was measured in cell pellets containing specified number of cells by MP-AES.

No reaction to SPIONdex infusion in a pig model of CARPA

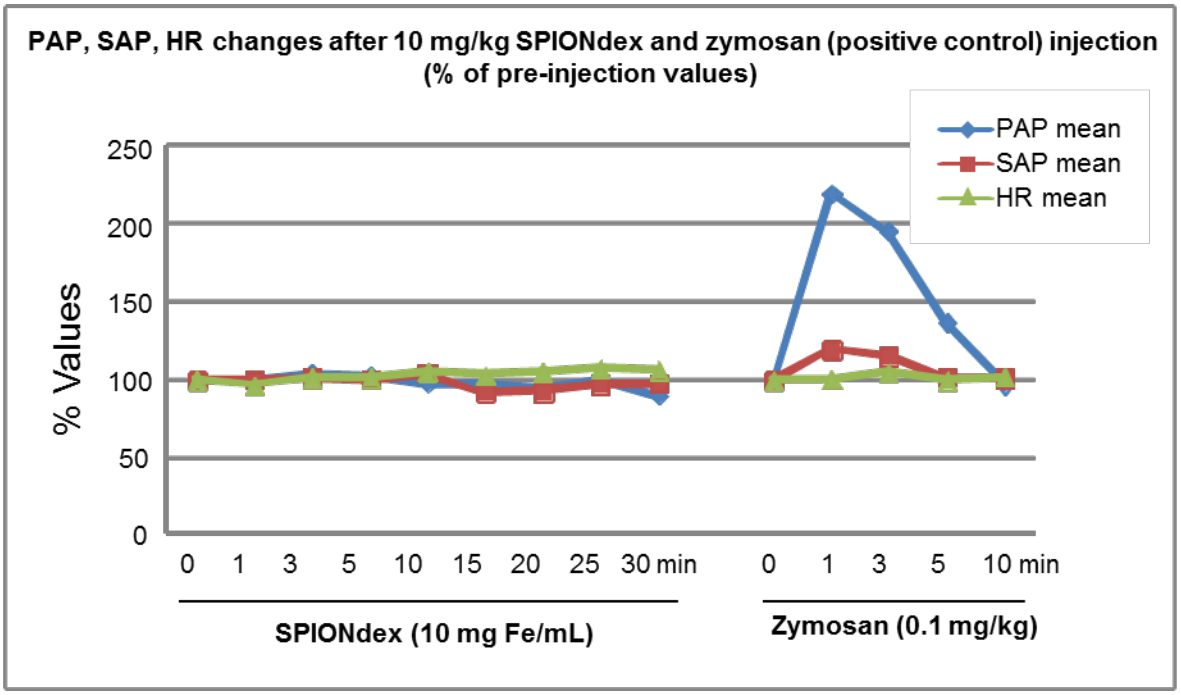
The cardiovascular changes in pigs upon intravenous bolus injection of SPIONdex (5 mg Fe/kg) were evaluated as described in Methods. Following the saline (negative control) injection, bolus of 5 mg Fe/kg was administered intravenously. Upon the injection of SPIONdex particles at 5 mg Fe/kg dose, no CARPA was observed (Suppl. Fig. 8A). The presence of full tachyphylaxis was confirmed by the injection of 2x higher dose 30 minutes

later, which also caused no reaction. A severe CARPA-reaction was observed by administration of 0.1 mg/kg zymosan (pos. control), as shown in Suppl. Fig. 8B.

A



B

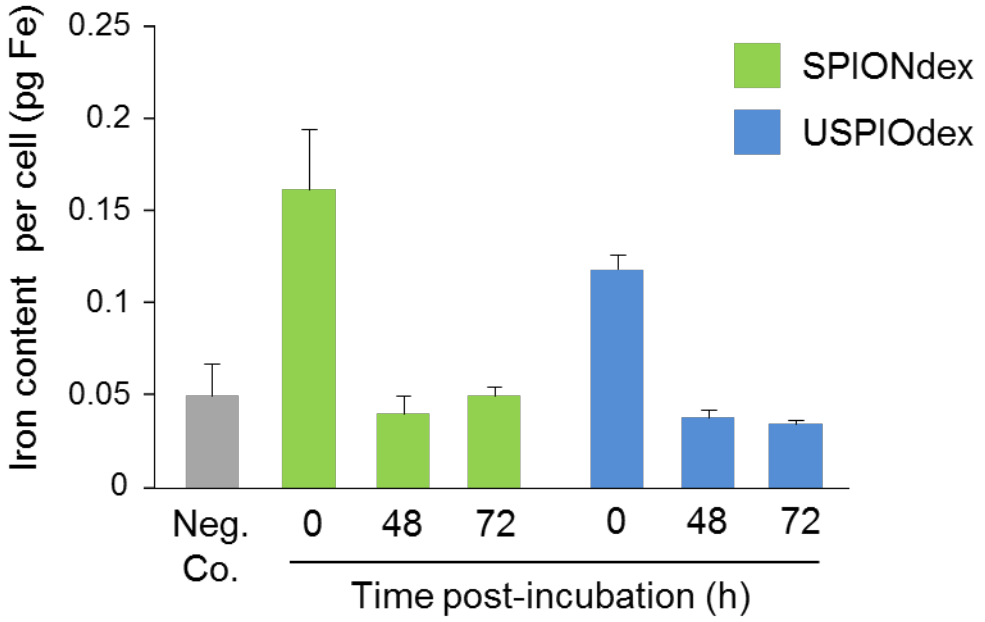


Suppl. Figure 8. Cardiovascular reaction to high dose of SPIONdex in a pig model of CARPA.
(A) Reaction to SPIONdex at 5 mg/kg dose; (B) Tachyphylaxis upon injection of 10 mg/kg dose 30 minutes later and severe CARPA reaction to zymosan (positive control). Saline (negative control), SPIONdex and zymosan were injected in the pigs per intravenous bolus

(<10 s). Pulmonary arterial blood pressure (PAP), systemic arterial blood pressure (SAP), and hear rate (HR) were continuously monitored for up to 30 min.

Endothelial clearance of SPION/USPIOdex

To evaluate the fate of internalized nanoparticles, HUVECs were incubated with SPIONdex or USPIOdex (at 200 µg Fe/cm^2) for 24 h. Following multiple washing steps with PBS, HUVECs were either harvested immediately (time 0h) or cultured in the absence of SPIONs for further 48 or 72 h. The cellular iron content was measured by MP-AES and compared to negative control sample (nanoparticle-untreated cells). As shown in Suppl. Figure 9, the incubation of ECs with SPION/USPIOdex for 24 h resulted in 2.5 to 3-fold increase in cell iron content, which returned to the untreated control levels within 48h post-incubation indicating a rapid clearance to these particles.



Suppl. Figure 9. Endothelial clearance of SPIONdex and USPIOdex.
ECs were grown to 90% confluence, followed by 24 h incubation with 200 µg Fe/mL of either SPIONdex (green bars) or USPIOdex (blue bars) and washing. Untreated Ecs served as negative control. Iron content was measured by MP-AES, in cell pellets containing specified number of cells, immediately post-incubation (time 0 h), and after 48 and 72 h.

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References

1. Unterweger H, Tietze R, Janko C, et al. Development and characterization of magnetic iron oxide nanoparticles with a cisplatin-bearing polymer coating for targeted drug delivery. *International journal of nanomedicine.* 2014;9:3659-3676.
2. Dobrovolskaia MA, Neun BW, Clogston JD, Grossman JH, McNeil SE. Choice of method for endotoxin detection depends on nanoformulation. *Nanomedicine (Lond).* 2014;9(12):1847-1856.
3. Dobrovolskaia MA, Neun BW, Man S, et al. Protein corona composition does not accurately predict hematocompatibility of colloidal gold nanoparticles. *Nanomedicine : nanotechnology, biology, and medicine.* 2014;10(7):1453-1463.
4. Dobrovolskaia MA, Patri AK, Simak J, et al. Nanoparticle size and surface charge determine effects of PAMAM dendrimers on human platelets in vitro. *Molecular pharmaceutics.* 2012;9(3):382-393.
5. Ilinskaya AN, Man S, Patri AK, et al. Inhibition of phosphoinositol 3 kinase contributes to nanoparticle-mediated exaggeration of endotoxin-induced leukocyte procoagulant activity. *Nanomedicine (Lond).* 2014;9(9):1311-1326.
6. Cicha I, Beronov K, Ramirez EL, et al. Shear stress preconditioning modulates endothelial susceptibility to circulating TNF-alpha and monocytic cell recruitment in a simplified model of arterial bifurcations. *Atherosclerosis.* 2009;207(1):93-102.
7. Zuehlke J, Ebenau A, Krueger B, Goppelt-Struebe M. Vectorial secretion of CTGF as a cell-type specific response to LPA and TGF-beta in human tubular epithelial cells. *Cell Commun Signal.* 2012;10.
8. Cicha I, Regler M, Urschel K, Goppelt-Struebe M, Daniel WG, Garlichs CD. Resveratrol Inhibits Monocytic Cell Chemotaxis to MCP-1 and Prevents Spontaneous Endothelial Cell Migration Through Rho Kinase-Dependent Mechanism. *J Atheroscler Thromb.* 2011;18(12):1031-1042.
9. Szebeni J. Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals. *Molecular immunology.* 2014;61(2):163-173.
10. Greish K, Thiagarajan G, Herd H, et al. Size and surface charge significantly influence the toxicity of silica and dendritic nanoparticles. *Nanotoxicology.* 2012;6(7):713-723.
11. Nabeshi H, Yoshikawa T, Matsuyama K, et al. Amorphous nanosilicas induce consumptive coagulopathy after systemic exposure. *Nanotechnology.* 2012;23(4).
12. Shaw AW, Pureza VS, Sligar SG, Morrissey JH. The local phospholipid environment modulates the activation of blood clotting. *The Journal of biological chemistry.* 2007;282(9):6556-6563.
13. Morrissey JH, Davis-Harrison RL, Tavoosi N, et al. Protein-Phospholipid interactions in blood clotting. *Thromb Res.* 2010;125:S23-S25.

14. Osterud B, Bjorklid E. Tissue factor in blood cells and endothelial cells. *Frontiers in bioscience.* 2012;4:289-299.
15. Matuszak J, Baumgartner J, Zaloga J, et al. Nanoparticles for intravascular applications: Physicochemical characterization and cytotoxicity testing. *Nanomedicine (Lond).* 2016;11(6).
16. Cicha I, Scheffler L, Ebenau A, Lyer S, Alexiou C, Goppelt-Struebe M. Mitoxantrone-loaded superparamagnetic iron oxide nanoparticles as drug carriers for cancer therapy: Uptake and toxicity in primary human tubular epithelial cells. *Nanotoxicology.* 2015:1-10.